Exhaled 8-Isoprostane as an *In Vivo* Biomarker of Lung Oxidative Stress in Patients with COPD and Healthy Smokers

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Most of the studies linking chronic obstructive pulmonary disease (COPD) with oxidative stress are *in vitro*, using invasive techniques, or measuring systemic oxidative stress. The aim of this study was to quantify oxidative stress in the lungs in patients with COPD and in healthy smokers, as reflected by 8-isoprostane concentrations in breath condensate. This is a noninvasive method to collect airway secretions. 8-Isoprostane is a prostaglandin-$F_{2\alpha}$ isomer that is formed *in vivo* by free radical-catalyzed peroxidation of arachidonic acid. We also studied the acute effect of smoking on exhaled 8-isoprostane in healthy smokers. Exhaled 8-isoprostane was measured by a specific enzyme immunoassay in 10 healthy nonsmokers and 12 smokers, 25 COPD ex-smokers, and 15 COPD current smokers. 8-Isoprostane concentrations were similar in COPD ex-smokers (40 ± 3.1 pg/ml) and current smokers (45 ± 3.6 pg/ml) and were increased about 1.8-fold compared with healthy smokers (24 ± 2.6 pg/ml, $p < 0.001$), who had 2.2-fold higher 8-isoprostane than healthy nonsmokers (10.8 ± 0.8 pg/ml, $p < 0.05$). Smoking caused an acute increase in exhaled 8-isoprostane by about 50%. Our study shows that free radical production is increased in patients with COPD and that smoking causes an acute increase in oxidative stress.

Oxidative stress is a major component of airway inflammation in chronic obstructive pulmonary disease (COPD) (1). An imbalance between oxidants and antioxidants may play an important role in the pathogenesis of COPD (1). Exhaled hydrogen peroxide, a biomarker of oxidative stress, is increased in breath condensate of patients with both stable and exacerbated COPD compared to healthy subjects (2). Plasma antioxidant capacity is decreased in the patients with acute exacerbations of COPD (3). Oxidative stress is also increased in chronic healthy smokers (4).

Isoprostanes are prostaglandin analogs produced by free radical-catalyzed peroxidation of arachidonic acid (5). Measurements of these compounds in exhaled breath condensate have several advantages over other quantitative indices of oxidative stress (5): isoprostanes (*1*) are chemically stable, (*2*) are formed *in vivo*, (*3*) are specific for lipid peroxidation, which is an important step in oxidative stress, (*4*) exert potent biological activity, which may be relevant to the pathophysiology of lung diseases, (*5*) are used to define the clinical pharmacology of antioxidants, and (*6*) are measured in exhaled breath condensate, which is likely to reflect oxidative stress in the lung (8). 8-Isoprostane, a member of the F2 isoprostane class, is increased in urine in patients with stable COPD (6). We have previously demonstrated an increase in 8-isoprostane in bronchoalveolar lavage (BAL) fluid of patients with interstitial lung diseases and in exhaled breath condensate of patients with asthma and cystic fibrosis (7–9). 8-Isoprostane is also increased in urine and BAL in patients with atopic asthma after antigen challenge (10).

Most of the studies linking COPD with oxidative stress are *in vitro* (1), using invasive techniques such as BAL fluid (11), or measuring systemic rather than lung oxidative stress (3, 6).

The aim of this study was to quantify oxidative stress in the lungs in patients with stable COPD (current and ex-smokers) and in healthy smokers as reflected by 8-isoprostane concentrations in exhaled breath condensate. Cigarette smoking is a major risk factor for COPD (1). We also studied the acute effect of smoking on lung oxidative stress in healthy smokers.

METHODS

Patients and Study Design

Two studies were conducted. In a cross-sectional study four groups of subjects were studied: 10 healthy nonsmokers, 12 healthy smokers, 25 patients with COPD who were ex-smokers, and 15 patients with COPD who were current smokers (Table 1). Patients with COPD attended the outpatient clinic at the Royal Brompton Hospital in London. Informed consent was obtained from all subjects. This study was approved by the Ethics Committee of the Royal Brompton Hospital and Harefield Trust. Study groups were matched for age (Table 1). Diagnosis of COPD was based on the British Thoracic Society guidelines (12). All the patients had airways obstruction with forced expiratory volume in 1 s (FEV_1) < 80% predicted and forced expiratory volume in 1 s/forced vital capacity (FEV_1/FVC) ratio < 70%, which did not change markedly over 2 mo, no spirometric response to bronchodilators (FEV_1 increase lower than 200 ml and 15% of baseline values), a history of chronic progressive symptoms such as dyspnea, cough, and wheeze, and a history of smoking. Patients with a history of atopy or positive skin prick testing for common inhaled allergens, and history of asthma or other respiratory diseases were excluded from the study. Chest radiography was carried out to exclude other respiratory diseases. Patients with systemic diseases, vascular disease, thrombosis, alcoholism, renal disease, and hepatic disease were excluded from the study. Patients with COPD were clinically stable with no worsening of symptoms within the previous 8 wk. FEV_1/FVC ratio was unchanged over a period of at least 8 wk before breath condensate collection. Eight COPD current smokers and 11 COPD ex-smokers were at stage I ($FEV_1 \geq 50\%$ predicted) according to the American Thoracic Society guidelines for COPD (13), and the others were at stage II (FEV_1 35 to 49% predicted). Healthy smokers, COPD ex-smokers, and COPD current smokers were matched for smoking habits and had a history of more than 20 pack-years (Table 1). Smoking status was checked by urinary cotinine levels (data not shown). Ex-smokers had stopped smoking for at least 6 mo. Healthy smokers and COPD current smokers refrained from smoking for at least 12 h before breath condensate collection. No subject had received vitamin supplements in the previous 4 wk. Twenty-two COPD ex-smokers were treated with inhaled (beclomethasone dipropionate 0.5–2 mg/d, budesonide 0.4–1.2 mg/d, fluticasone 0.5–2 mg/d) and/or oral corticosteroids (prednisolone 5–10 mg/d). Twelve COPD current smokers were treated with inhaled (beclomethasone dipropionate 0.4–1 mg/d,

(*Received in original form January 18, 2000 and in revised form March 15, 2000*)

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Am J Respir Crit Care Med Vol 162. pp 1175–1177, 2000
Internet address: www.atsjournals.org

TABLE 1
STUDY OF FOUR GROUPS OF SUBJECTS*

	Healthy Nonsmokers	Healthy Smokers	COPD Ex-smokers	COPD Current Smokers
n	10	12	25	15
Age, yr	63 ± 4	60 ± 3	66 ± 3	62 ± 2
Sex, F/M	5/5	5/7	13/12	8/7
FEV_1, L	4.40 ± 0.25	3.90 ± 0.30	1.56 ± 0.23‡	1.37 ± 0.16‡
FVC, L	4.60 ± 0.36	4.33 ± 0.34	2.78 ± 0.27‡	2.50 ± 0.27‡
FEV_1, % pred	93.0 ± 4.3	87.3 ± 4.1	54.1 ± 3.1‡	53.8 ± 3.7‡
FVC, % pred	97.2 ± 3.8	93.4 ± 3.3	82.7 ± 3.3†	78.3 ± 4.1†
FEV_1/FVC, %	95.6 ± 2.7	90.7 ± 3.1	56.1 ± 3.0‡	54.6 ± 2.5‡
Pack-years	0	34 (20–80)	40 (20–75)	40 (20–82)
Therapy				
Inhaled steroids	No	No	22/25	12/15
Oral steroids	No	No	5/25	4/15
Theophylline	No	No	10/25	5/15
β_2-agonists	No	No	19/25	8/15

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; FEV_1 = forced expiratory volume in 1 s; FVC = forced vital capacity.

* Data are expressed as means ± SEM. Pack-years are expressed as median with range in parentheses.

† $p < 0.05$, ‡ $p < 0.01$ compared with healthy nonsmokers.

budesonide 0.4–1.6 mg/d, fluticasone 1 mg/d) and/or oral corticosteroids (prednisolone 5–10 mg/d). Inhaled β-adrenergic agonists, and theophylline were also used (Table 1).

In a second study, the acute effect of cigarette smoking was investigated in 12 healthy smokers (age 37 ± 8 yr, six males, FEV_1 97 ± 2.7% predicted, pack year 8 ± 2). Subjects were asked to refrain from smoking for at least 12 h before starting the study. We collected a sample of breath condensate to measure 8-isoprostane. We asked the subjects to smoke two cigarettes consecutively. We collected another breath condensate sample after 15 min. The measurements were repeated after 5 h.

Pulmonary Function

Spirometry was measured using a dry spirometer (Vitalograph Ltd, Buckingham, UK), and the best value of the three maneuvers was expressed as an absolute value (liters) and as a percentage of the predicted value.

Measurement of Exhaled 8-Isoprostane

Breath condensate samples were collected using a specially design condensing chamber (Ecoscreen; Jaeger, Hoechberg, Germany). Exhaled air entered and left the chamber through one-way valves at the inlet and outlet, thus keeping the chamber closed. Subjects breathed tidally through a mouthpiece connected to the condenser for 15 min while wearing noseclips. A −20° C temperature inside the condensing chamber throughout the collection time produced immediate sample freezing. Approximately 2.5 ml of breath condensate was collected. 8-Isoprostane concentrations in breath condensate were measured by a specific enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI) as previously described (8). The antiserum used in this assay has a 100% cross-reactivity with 8-isoprostane, 0.2% with $PGF_{2\alpha}$, $PGF_{3\alpha}$, PGE_1, and PGE_2, and 0.1% with 6-keto-$PGF_{1\alpha}$. The detection limit of the assay is 4 pg/ml. Possible influence of ventilation rate on 8-isoprostane concentration in breath condensate and saliva contamination of breath condensate were excluded (9). The intraassay and interassay variability were ± 5% and 6%, respectively.

Statistical Analysis

One-way analysis of variance (ANOVA) with Newman–Keuls test for multiple comparisons was used to compare groups. Linear regression analysis was used to assess the relationship between 8-isoprostane concentrations in breath condensate and FEV_1. All data were expressed as means ± standard error of mean and significance was defined as a p value of < 0.05.

RESULTS

Clinical data of healthy subjects and patients with COPD are summarized in Table 1. 8-Isoprostane levels were detectable (10.8 ± 0.8 pg/ml) in breath condensate of healthy nonsmokers, and were increased in healthy smokers (24.3 ± 2.6 pg/ml, $p < 0.05$), COPD ex-smokers (39.9 ± 3.1 pg/ml, $p < 0.001$), and COPD current smokers (45.3 ± 3.6, $p < 0.001$) (Figure 1). 8-Isoprostane was higher in COPD ex-smokers and COPD current smokers compared with healthy smokers ($p < 0.001$) (Figure 1). 8-Isoprostane levels in COPD ex-smokers and current smokers were similar (Figure 1). There was no correlation between 8-isoprostane and age, sex, FEV_1, FVC, FEV_1/FVC ratio, and history of smoking in pack-years.

In healthy smokers, 8-isoprostane concentrations in breath condensate increased 15 min after smoking compared with basal levels (32.3 ± 2.8 pg/ml versus 20.7 ± 1.8 pg/ml, $p < 0.03$) (Figure 2). Although not statistically significant, there was a trend to increased 8-isoprostane concentrations after 5 h (28.9 ± 4.0 pg/ml versus 20.7 ± 1.8 pg/ml, $p < 0.073$) (Figure 2).

DISCUSSION

8-Isoprostane is formed *in vivo* by free radical peroxidation of arachidonic acid (5). Measurement of these compounds in biological fluids may provide a quantitative index of oxidative stress *in vivo* (5). Isoprostane may also be produced by cyclooxygenase (COX)-1 and COX-2 activation in some cells and tissue *in vitro* (5). However, *in vivo* 8-isoprostane enzymatic synthesis in humans seems to be negligible (6). Oxidative stress is a major component of airway inflammation in COPD (1). Cigarette smoking is the major risk factor for COPD (1) and causes oxidative damage (4).

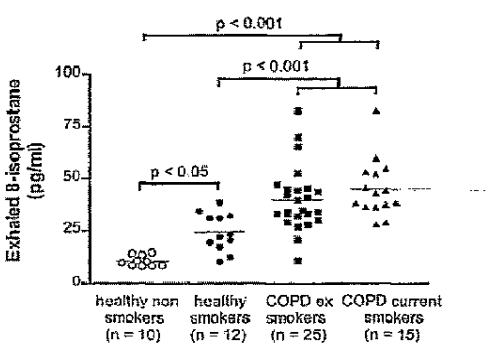

Figure 1. 8-Isoprostane concentrations in breath condensate in healthy nonsmokers, healthy smokers, patients with COPD who are current smokers, and patients with COPD who are ex-smokers. Mean values are shown by *horizontal bars*.

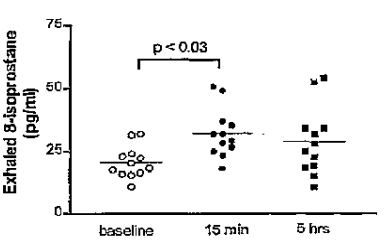

Figure 2. 8-Isoprostane concentrations in breath condensate in healthy smokers before smoking and after 15 min and 5 h from smoking. Mean values are shown by *horizontal bars*.

In this study, we showed that oxidative stress, as reflected by 8-isoprostane concentrations in breath condensate, is increased in patients with COPD (both current and ex-smokers). 8-Isoprostane levels were higher in patients with COPD (current and ex-smokers) compared with healthy nonsmokers. Healthy smokers had 8-isoprostane concentrations over 2-fold higher than healthy nonsmokers. The effect of smoking on lipid peroxidation is acute, as shown by an increase in breath condensate 8-isoprostane concentrations by about 50% over baseline 15 min after smoking, and there is a trend toward increased levels after 5 h. Our results are in contrast with those reported by Morrow and coworkers who did not find any short-term effect of smoking on plasma levels of F2-isoprostanes (4). This discrepancy could be explained on the basis of the differences of the two compartments, with breath condensate mainly reflecting lipid peroxidation in the lungs, whereas plasma concentrations reflect systemic oxidative stress. Smoking causes an immediate increase in 8-isoprostane levels in breath condensate, but the intensity of the oxidant injury may not be sufficient to affect plasma concentrations. On the other hand, it has been shown using the same analytical technique we used that ozone exposure causes an 80-fold increase in 8-isoprostane in airway lavage without affecting the plasma levels of the compound (14).

COPD and healthy current smokers were matched for smoking habits, but the former had 1.9-fold higher 8-isoprostane levels, indicating a higher level of oxidant stress in patients with COPD. COPD ex-smokers had stopped smoking for at least 6 mo, yet 8-isoprostane levels were 1.6-fold higher than in healthy current smokers. This may reflect the continuing oxidative stress and inflammation in ex-smokers with COPD (15). There was no difference in exhaled 8-isoprostane concentrations between COPD ex-smokers and current smokers. Patients with COPD were all heavy smokers (pack-years > 20). In these patients, lipid peroxidation due to chronic smoking might have reached a point at which smoking cessation has little, if any, effect. The lack of correlation between 8-isoprostane and lung function tests is in line with a previous study (6). Interindividual differences in susceptibility to the same levels of oxidative stress or mechanisms different from oxidative stress contributing to airway inflammation could explain these findings.

Most of the patients with COPD in our study were treated with steroids. This made it impossible to investigate any effect of steroids on 8-isoprostane in patients with COPD. Double-blind placebo-controlled studies are required to definitively establish the effects of steroid treatment on exhaled 8-isoprostane. Our approach was not able to ascertain the cellular source of 8-isoprostane for which *in vitro* studies are required. It is not known if 8-isoprostane is actively involved in the pathogenesis of COPD. Inflammation and to a lesser extent bronchoconstriction contribute to airflow obstruction in COPD (13). 8-Isoprostane causes contraction of human bronchi *in vitro* and plasma exudation in guinea pigs *in vivo* (16). If these effects occur in humans *in vivo*, isoprostane could be functionally involved in COPD inflammation.

In conclusion, we have shown that oxidative stress is increased in patients with stable COPD as reflected by an increase in exhaled 8-isoprostane. Measurement of this biomarker in the exhaled air may provide a useful, sensitive, and noninvasive approach in dose-finding studies with antioxidants.

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